pH (for example, pH 8 to 11), the solution is incubated at an adequate temperature. Alternatively, the solution is incubated at a temperature at which the toxic activity is generally lost (for example, at a temperature of 40°C or higher). Otherwise, the toxin is sonicated at an adequate wavelength, or irradiated with electromagnetic wave. The residual toxic activity of the sample is assayed before, during and after the treatment. If desired, the attenuated toxin is re-incubated at an adequate temperature, for example, at 37°C, to secure that the toxic activity does not recover. If the activity of enhancing immunity is confirmed to be sufficiently high, then the preparation of attenuated toxin is completed. The chemical and physical treatments can be properly combined for attenuation.

Method for verifying residual toxin activity

Residual toxic activity can be assayed by any conventional method (see, for example, "Handbook for vaccination," Ed. Research Association, National Institute of Health of Japan, Maruzen, 1994; "Protein toxin," 1st and 2nd volumes, Ed., R. Tamura et al., Kodansha Scientific, 1972, etc.). The methods can be categorized as follows: enzyme activity assays, physiological-response assays using animal cells, physiological-response assays using animals, and evaluation methods utilizing survival and death of experimental animals, etc. The specific assay method to be used depends on the type of toxin. The following examples describe representative indexes for selecting such a method. Specific procedures for these assays are known. The comparison of toxic activity is not limited to these indexes, but as the matter of course the comparison of toxin activities before and after the attenuation should be carried out using the same index.

(Bacterial toxin)

Cholera toxin, heat-labile toxin of pathogenic *E. coli*

ADP-ribosyltransferase activity
Accumulation of cAMP
Y-1 cell morphologic transformation
Weight loss in mouse
Elongation of CHO cell

Pertussis toxin
- ADP-ribosyltransferase activity
- Elongation of CHO cell
- Leukocyte increasing activity
- Histamine sensitizing activity
- Weight loss in mouse
- Elongation of CHO cell

Diphtheria toxin
- ADP-ribosyltransferase activity
- Survival of guinea pig
- Rabbit cutaneous sensitization activity

Tetanus toxin
- ADP-ribosyltransferase activity
- Death of guinea pig
- Rabbit cutaneous sensitization activity

Staphylococcus α toxin, staphylococcus β toxin
- Hemolytic activity

Shiga toxin
- Hemolytic activity

Pseudomonas enterotoxin A
- ADP-ribosyltransferase activity

Vibrio parahaemolyticus thermostable hemolytic toxin
- ADP-ribosyltransferase activity
- Hemolytic activity

(Fungus toxin)

Canditoxin, fumigatoxin,
- Mouse killing activity

(Animal toxin)

Snake venom
- Cholinesterase activity
- Cholinesterase-inhibiting activity
- Phospholipase A2 activity
- Blood coagulation activity
- Platelet aggregation activity
- Anticomplement activity

Bee toxin

N-acetyl glucosaminidase activity

Hyaluronidase activity

In assaying toxin activity, even when the residual toxic activity is not detected by one method, a different result can be obtained by an alternate method. For example, there was a case where the toxic activity was detected by Y-1 cell morphologic transformation test although substantially no residual activity of attenuated cholera toxin was recognized by ADP-ribosyltransferase activity (for example, K. Komase et al., Vaccine 16, 248-254, 1998). In another case, although ADP-ribosyltransferase activity was substantially not detectable in a recombinant mutant of *E. coli* heat-labile toxin in which the amino acid residue was replaced with lysine residue at amino acid position 7 from the N terminus, the attenuated toxin induced diarrhea. In other words, the toxic activity of *E. coli* heat-labile toxin can be represented by ADP-ribosyltransferase activity, but there is a second minor toxic activity that is expressed by a different mechanism. It is thought that when the first activity is markedly reduced in the recombinant mutant, then the second toxic activity increases relatively and as a consequence the second one becomes detectable. In general, assay methods with living cells or living bodies tend to show higher sensitivity in detecting toxic activity. The above-mentioned mechanism is considered to be involved in this detection.

Accordingly, in general, the comparison of toxic activity can be performed more reliably by using a sensitive method based on the response of living cells or living bodies. It is also preferable to evaluate the residual toxic activity by multiple assaying methods. In Examples described herein, the residual toxic activity of cholera toxin was evaluated by the combined use of an assay for receptor-binding activity, a Y-1 cell morphologic transformation test, a mouse foot swelling test, and a method involving the intraperitoneal administration to a mouse. Enzyme immunoassay (ELISA) using anti-toxin antibody is also a useful index representing the toxic activity. With any method, the most important thing is to reduce the toxic activity to at least one-two thousandth that of the natural toxin, in principle, based on an index that directly reflects the toxic activity.